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Respiratory Carcinogenicity of Diesel Fuel Emissions

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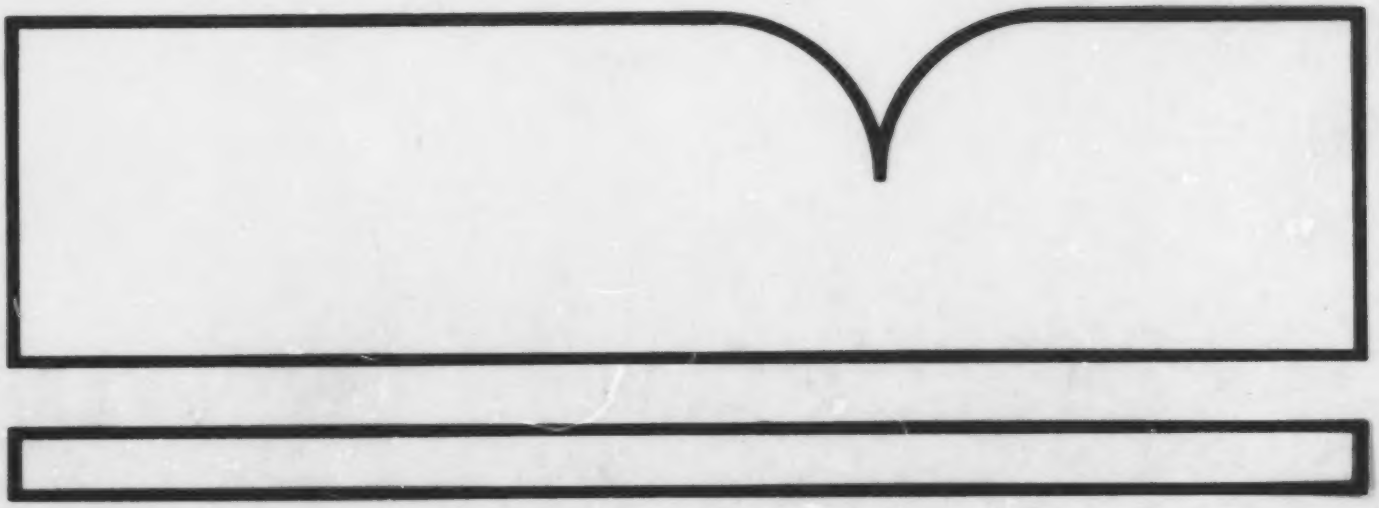

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RESPIRATORY CARCINOGENICITY OF DIESEL FUEL EMISSIONS

by

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16. ABSTRACT

The objective of this program was to evaluate the possible respiratory carcinogenic effects of diesel fuel emission particles and organic extracts of these particles in suitable animal models. Because of our previous experience in the use of the Schreiber method for localized tumor induction and the rapid response time observed in this model with certain known direct acting carcinogens it was planned to initiate our studies with this model. Subsequently studies were to be initiated using the Saffiotti technique for intratracheal instillation for evaluation of life-time effects.

This report also describes certain studies which were initiated but not completed due to decisions concerning program relevance and to choices made concering the expenditure of available program funds.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This grant was initiated to provide information relating to the potential respiratory carcinogenicity of diesel emission products.

F. Gordon Hueter
Director
Health Effects Research Laboratory

ABSTRACT

The objective of this program was to evaluate the possible respiratory carcinogenic effects of diesel fuel emission particles and organic extracts of these particles in suitable animal models. Because of our previous experience in the use of the Schreiber method for localized tumor induction and the rapid response time observed in this model with certain known direct acting carcinogens it was planned to initiate our studies with this model. Subsequently studies were to be initiated using the Saffiotti technique for intratracheal instillation for evaluation of life-time effects.

The program was initially planned for a three-year period with the major emphasis during the first year to be placed on short-term studies with the Schreiber model. Shortly after program initiation a scientific review meeting was held on the Diesel Emission Health Effects Research Program (December 12, 13, 1978 at Arlington, Virginia). As a result of this meeting and other program considerations concerning risk assessment utility, emphasis under this grant was gradually shifted to the utilization of the Saffiotti intratracheal instillation model. In addition, program plans were modified to include the assessment of coke oven extract, roofing tar extract and cigarette smoke condensate on which a human epidemiologic data base existed.

As a result of those considerations this grant was terminated after one year and an extensive study utilizing the Saffiotti technique was initiated under Cooperative Agreement No. R806929-01-0 on September 1, 1979. Some of the initial dose range studies which were started on the original program were continued and completed under the successor program and have been reported upon as required.

This report also describes certain studies which were initiated but not completed due to decisions concerning program relevance and to choices made concerning the expenditure of available program funds.

This report was submitted in fulfillment of Grant No. R806326-01-1 under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period from October 2, 1978 to September 30, 1979, and work under this grant was completed as of September 30, 1979.

CONTENTS

	Page
Disclaimer	ii
Foreword	iii
Abstract	iv
Tables	vi
Figures	vi
Abbreviations	vii
Acknowledgments	viii
1. Introduction, Conclusions and Recommendations	1
2. Materials and Methods	2
3. Experimental Procedures	10
Localized Treatment with DP Extracts	10
Intratracheal Instillation Experiments with the Saffiotti Method	10
Lung Clearance of Diesel Particles	10
4. Results and Discussion	13
Localized Treatment with DFE (Schreiber Method)	13
Dose-Range Study of Diesel Emission Particles	15
Lung Clearance of Diesel Particles.	17
References	20
Appendices	
A. Safety Plan for Handling of Carcinogens and Test Materials.	21
B. Protocol for the Collection and Submission of Necropsy Specimens (L6109).	24

TABLES

Number		Page
1	Particle Size Distribution of the "As Received" DP (Cumulative Number % Greater Than the Stated Diameter.	3
2	Particle Size Distribution of Diesel Particle and Ferric Oxide Suspensions (Cumulative Number % Greater Than the Stated Diameter.	6
3	Assay of 1.0 ml Aliquots of Typical Suspensions.	7
4	Percent Increase in Body Weight and Percent Death in Hamsters Administered Diesel Fuel Extract by Localized Intratracheal Instillation	14
5	Dose-Range Study to Determine Toxicity of Diesel Fuel Particles Administered by the Saffiotti Technique	16

FIGURES

Number		Page
1	Experimental Design for Dose-Range Study to Determine Toxicity of Diesel Fuel Extract Using the Localized Intratracheal Instillation Technique	11
2	Experimental Design for Dose-Range Study to Determine Toxicity of Diesel Fuel Extract Using the Saffiotti Intratracheal Instillation Technique	12

LIST OF ABBREVIATIONS

BA -- Benz(a)anthracene

BP -- Benzo(a)pyrene

DFE -- Diesel Fuel Emission Extract

DMBA -- Dimethylbenz(a)anthracene

DMSO -- Dimethyl Sulfoxide

DP -- Diesel Fuel Emission Particles

ETOH -- Ethyl Alcohol

Fe_2O_3 -- Ferric Oxide

GEL -- Gelatin

MNU -- Methylnitrosourea

PG -- Propylene Glycol

ACKNOWLEDGMENTS

The diesel fuel exhaust particles and the dichloromethane extract of exhaust particles used in this program were supplied by the Environmental Sciences Research Laboratory, EPA, Research Triangle Park, North Carolina through the courtesy of Drs. Ronald Bradow, Roy B. Zweidinger and Silvestre Tejade.

SECTION 1

INTRODUCTION, CONCLUSIONS, AND RECOMMENDATIONS

This grant was initiated to provide information relating to the potential respiratory carcinogenicity of diesel emission products. Emphasis was placed on the Schreiber model for localized tumor induction since it offered the possibility of a more rapid determination of adverse effects. Following a presentation at a Diesel Emission Health Effects Program meeting in December of 1978 and subsequent program discussions it was decided to deemphasize work using the Schreiber model and to initiate dose range toxicity studies by the Saffiotti intratracheal instillation technique.

This decision was reached for three primary reasons.

1. The Saffiotti method offers a proven and widely accepted model for inhalation effects.

2. Total dose administered is known with the Saffiotti technique but not for the Schreiber method.

3. The Saffiotti method is ideal for the evaluation of diesel emission particles which cannot be tested using the Schreiber technique.

Since EPA's program for the evaluation of the health effects of diesel fuel emissions was aimed at developing a series of potency comparisons between diesel emissions and other materials on which there existed an epidemiologic data base, it was recommended that this respiratory carcinogenesis study also include an evaluation of these additional materials. Therefore the remainder of the first year of this grant was devoted to initiating dose range studies with diesel emission particles using the Saffiotti method.

This grant was terminated after the first year and the dose range studies were incorporated into and completed under a Cooperative Agreement (R806929-01-0) which was entered into on September 1, 1979.

Results of these studies were summarized in a series of quarterly progress reports (IITRI Report Nos. L6109-1 through 3). The results of studies which were in progress at the time this grant was terminated were summarized in reports on the successor program (IITRI Report Nos. L6119-1 and L6119-2).

SECTION 2

MATERIALS AND METHODS

TEST MATERIALS

Diesel Particle and Diesel Particle-Ferric Oxide Suspensions

Diesel fuel exhaust particles (DP) were supplied to IITRI as dry loose powders packed under nitrogen, in dry ice. Exhaust from several runs of the EPA's 350 Oldsmobile diesel test engine were collected on a single teflon coated glass fiber filter. The exhaust particles were then scraped from the filter surface to produce the loose powders supplied to IITRI.

Characteristics---

Optical microscopical examination of the powder indicated no significant contamination of the DP by teflon and glass fibers. Microscopical examination also indicated geometric particle sizes were too large for intratracheal instillation. Particle morphologies indicated that aggregation of the individual submicrometer carbon particles comprising the exhaust had occurred both on the collection filter and before particles reached the filter. The presence of large diameter (up to 70 μm) hollow carbon spheres indicated some aggregation occurred before deposition on the filter. The predominant aggregate morphology -- flakes ranging in thickness from 0 to 0.5 μm - - could have been formed both before and after deposition on the filter. The size distribution of the DP aggregates "as received" is shown in Table 1.

Attempts to disperse the powder into its constituent submicrometer particles by conventional techniques for deagglomeration further confirmed the conclusion that the DP observed microscopically were indeed aggregates rather than agglomerates. Further microscopical examination revealed that the only way the powder could be dispersed into the individual exhaust particles was by dissolving the condensed organic species (extractable materials) which formed a cementing agent that held the ultrafine carbon particles in sheets and hollow spheres. Since microscopical analysis indicated that some of the aggregates had been formed before the exhaust was captured on the filter the most logical approach to reducing the particle size distribution of the powder to a range suitable for intratracheal instillation was to crush the aggregates.

TABLE 1. PARTICLE SIZE DISTRIBUTION OF THE "AS RECEIVED" DP
(CUMULATIVE NUMBER % GREATER THAN THE STATED DIAMETER)

Geometric[1] Diameter μm	Aerodynamic[2] Diameter μm	Greater Than Stated Diameter (No. %)
0	0	100
1.0	0.4	83.9
3.0	2.0	54.3
5.5	2.6	21.9
8.0	3.4	10.2
10.5	4.1	5.4
13.0	4.6	2.3
15.5	5.3	1.0
18.0	5.8	0.5
20.5	7.3	0.2

[1]Geometric Diameter is the diameter of a circle having the same projected area as the DP.

[2]Aerodynamic Diameter is the diameter of a sphere having the same volume as the DP, assuming an average thickness of 0.3 μm for the DP aggregates.

Suspension Preparation---

Diesel exhaust particles (DP) were to be prepared as suspensions in gelatin-saline, with and without ferric oxide (Fe_2O_3). Concentrations of DP were to be 5, 3 and 1 mg per 0.2 ml of suspension. DP suspensions with Fe_2O_3 contained equal mass concentrations (5, 3 and 1 mg/0.2 ml) of DP and Fe_2O_3. Particle sizes below 10 μm were required.

The low density of the DP powder, its wide particle size range (0-150 μm), its hydrophobic and electrostatic natures, and sterility requirements precluded the possibility of dry grinding the powder to the desired size range, before suspension in gelatin-saline. Rather, size reduction and suspension of the reduced particles were best accomplished simultaneously. This was achieved by use of a ball milling technique.

Milling apparatus consisted of wide mouth, cylindrical, pyrex glass jars with silicon-rubber lined bakelite caps; 3-5 mm solid pyrex glass beads; and a variable speed roller. The milling jars were half-filled with glass beads before the appropriate volume of saline and masses of gelatin and iron oxide were added. The jars were then loosely covered with the bakelite caps and autoclaved. After the jars had cooled, the DP were added. Because of the hydrophobic nature of the DP, they were first mixed with a wetting agent, propylene glycol (PG) (7% by volume of the total suspension). The resulting paste was then quickly transferred to the sterilized milling jar. The control suspensions also contained propylene glycol and were ball milled.

A milling time of 10 days was required to reduce 95% by mass of the DP aggregates below 10 μm.

The completed suspension was separated from the milling balls with a buchner funnel. The funnel as well as the collecting vessels were all autoclaved before use.

The DP tended to agglomerate in the suspension. The agglomerates in fresh suspensions could be redispersed by simple shaking. However, aged suspensions required more vigorous deagglomeration methods, and suspensions over 4 weeks old could not be deagglomerated. Therefore, suspension volumes sufficient for three weeks of instillation were prepared at one time. To prevent particle size distribution changes with repeated deagglomerations, suspensions were supplied in vials containing one week's worth of material which was stored at -70°C until administered.

Suspension Characterizations---

Suspensions supplied for intratracheal instillations were checked for sterility, particle size distribution and mass concentrations of suspended particles. Standard culture techniques were used to check each vial supplied for sterility. Optical microscopical examination of a small aliquot of each suspension consisted of determining the largest aggregate size present and estimating the per cent of oversized particles present. Previous work with benzo(a)pyrene ball milled suspensions has indicated that particle size distributions are consistent from batch to batch. Therefore, detailed

particle size distribution analyses were performed by image analysis on only three of the batches prepared. Table 2 lists typical particle size distributions for DP and Fe_2O_3 suspensions. Note that the additions of the Fe_2O_3 served to further reduce DP particle sizes. No significant differences in particle size distributions were noted between the various concentrations prepared.

Assays of suspended particle concentrations were performed by several different methods. Total suspended particle concentrations were determined by filtering aliquots of suspension through tared 0.05 μm pore size membrane filters. Aliquot size used was 0.5 to 1.0 ml, depending upon the suspended particle concentration. For the DP suspensions, alone, this assay method provided the concentration of the DP particles in suspension. For the DP-Fe_2O_3 suspensions, however, further assaying was required to provide mass concentrations of each particle type. Iron concentration was determined by digestion of a 0.2 ml aliquot of suspension in hydrochloric acid; the dissolved iron was separated from the insoluble carbon particles by filtration. Iron concentration in the filtrate was determined spectrophotometrically. This assay method was specifically used to determine delivered dose concentrations. DP concentrations in the DP-Fe_2O_3 suspensions were determined by low temperature ashing the filter containing both the DP and Fe_2O_3 particles (from the total suspended particle assay). The total mass lost during ashing was the mass of the filter plus the mass of the carbonaceous diesel exhaust particles.

Aliquots of each batch prepared were assayed by filtration and ashing techniques. Table 3 presents the typical results of these two assay methods. Reproducibility of the filtration technique was determined to be ± 3%. The two different assaying methods produced DP concentrations with ± 5% of each other.

Actual DP concentrations were consistently lower than the theoretical concentrations, by 7 to 10%. The low concentrations can be attributed to two factors: solvation of some components of the DP (up to 5% of the total DP mass according to the literature); and the difficulty in completely transferring a suspension from one vessel to another, particularly during separation of the grinding media from the suspension.

Known Carcinogens

Certain experimental protocols required the administration of known carcinogens to test hamsters. BP, BA, DMBA and MNU were handled with the precautions described in the appended program Safety Plan (Appendix A).

TABLE 2. PARTICLE SIZE DISTRIBUTION OF DP AND DP-FE_2O_3 SUSPENSIONS (CUMULATIVE NUMBER % GREATER THAN THE STATED DIAMETER

Geometric[1] Diameter μm	Aerodynamic[2] Diameter μm	Greater Than Stated Diameter No. % of DP	No. % of DP + Fe_2O_3
0	0	100	100
1.0	0.4	83.7	84.9
3.0	2.0	48.2	44.7
5.5	2.6	20.5	19.7
8.0	3.4	7.9	8.2
10.5	4.1	2.6	2.1
13.0	4.6	1.0	0.7
15.5	5.3	0.3	0.2
18.0	5.8	0.1	0.1
20.5	7.3	0	0

[1]Geometric Diameter is the diameter of a circle having the same projected area.

[2]Aerodynamic Diameter is the diameter of a sphere having the same volume as the particles, assuming a thickness of 0.3 μm for the DP.

TABLE 3. ASSAY OF 1.0 ML ALIQUOTS OF TYPICAL SUSPENSIONS

	Theoretical Masses, mg			Assay By Filtration Masses, mg			Assay By Ashing		
Suspension*	DP	Fe_2O_3	Total	DP	Fe_2O_3	Total	DP	Fe_2O_3	Total
DP-Hi	25	-	25	22.8	-	22.8	23.8	-	23.8
DP+FE-Hi	25	25	50	-	-	50.7	24.4	26.3	50.7
DP-Med	15	-	15	13.9	-	13.9	13.3	-	13.3
DP+Fe-Med	15	15	30	-	-	29.6	13.8	15.8	29.6
DP-Lo	5	-	5	5.3	-	5.3	5.3	-	5.3
DP+Fe-Lo	5	-	10	-	-	9.4	4.4	5.0	9.4

*Hi = 5 mg/0.2 ml
Med = 3 mg/0.2 ml
Lo = 1 mg/0.2 ml

ANIMALS

Male Syrian Golden hamsters used in these studies were purchased from ARS Sprague-Dawley, Madison, Wisconsin or Engle Labs, Farmersburg, Indiana at 4 to 5 weeks of age. Upon arrival and inspection for general state of health, animals were housed three per cage in polycarbonate cages (18 1/2 x 10 x 8 1/2 in.). Cages, bedding, water bottles and food were changed twice weekly. Bedding consisted of Ab-Sorb-Dri or Pine-Dri and food (Wayne Blox) and tap water were provided *ad libitum*. Animal rooms were lighted with fluorescent lights on a 12-hour light/dark cycle. Animal room temperature was maintained at 20°C to 24°C with ambient relative humidity of 30 to 70%. All animals were quarantined for a period of at least two weeks prior to study initiation. During this time the health status of the hamsters was evaluated by physical examinations, gross necropsy and routine diagnostic microbiological workup. No abnormal pathology was observed in the quarantine hamsters.

Animal Identification

Animals were randomized into group sizes required by the experimental design. Hamsters were identified uniquely within each experiment by group, cage and individual animal number. Individual identification was achieved by ear punching of the hamsters. Individual cage cards contained group, cage and individual animal information.

Observations

Animals were observed twice daily for signs of toxicity or morbidity. Once weekly hamsters were weighed and given a physical examination. Animals that were moribund were isolated and observed more closely. Hamsters were allowed to die spontaneously, killed when moribund or sacrificed as specified by protocol. Individual necropsy forms were prepared with results of gross examination. Selected tissues and any lesions were submitted for histopathologic evaluation. A typical protocol for necropsy is presented in Appendix B.

INTRATRACHEAL INSTILLATION (SAFFIOTTI TECHNIQUE)

The method used is that previously described by Saffiotti and co-worker (1,2). Before each intratracheal instillation the hamster is anesthetized with halothane using an Airco Veterinary Anesthesia Machine, Heedbrink Model 960 (3). When the righting reflex is lost the animal is hung on a slanted board, its back on the board and its mouth kept open by hanging the lower incisor teeth on a wire hook, while the upper incisors are retained by a tight rubber band. A volume of 0.2 ml of the test material is drawn into a 0.25 tuberculin syringe. The syringe is fitted with a blunt 19 gauge needle about 3 in. long and bent at a 135° angle at 45 mm from the tip. A clear view of the pharynx is provided by a headlight worn by the operator The tongue is pulled outward with a forceps and the rhythmic opening and

closing of the vocal cords observed. The blunt end of the needle is inserted into the tracheal lumen past the open vocal cords the moment they are open. The needle is pushed almost to the bottom of the trachea and the suspension gently injected. The hamster is kept on the board for a minute to make certain no suspension is regurgitated.

LOCALIZED INTRATRACHEAL INSTILLATION

The device used for localized treatment of the hamster trachea was originally reported by Schreiber *et al* (4,5) and modified in our laboratory (6). A catheter fabricated of stainless steel is introduced into the trachea and 0.5 ml of the material under study is expressed from the outer tubes of the catheter. Simultaneously the inner tube of the catheter assembly is extended an additional 5 mm further into the trachea. The inner tube is attached to a vacuum system and the test solution is reabsorbed through the tip of the inner catheter. The test solution is applied for a period of five seconds during which period the vacuum exhaust is also in operation. The vacuum continues for an additional two seconds following which the catheter is withdrawn.

SECTION 3

EXPERIMENTAL PROCEDURES

GENERAL CONSIDERATIONS

Two general classes of experiments were carried out under this program. One series utilized a procedure for localized treatment of the trachea (6) and the other employed the Saffiotti model for intratracheal instillation. In addition a pilot study to explore the respiratory tract retention of a single dose of diesel particles was carried out.

Localized Treatment with Diesel Particle Extracts

Three initial experiments were carried out using this technique in order to determine dose ranges of diesel extract suitable for future experiments. A number of solvent mixtures including dimethyl sulfoxide, propylene glycol, ethanol and saline were investigated for their ability to solubilize the extract at desired concentrations. A typical experimental design is illustrated in Figure 1.

Intratracheal Instillation Experiments with the Saffiotti Method

A dose-range study of DP to determine suitable doses for the conduct of the chronic studies was carried out as shown in Figure 2. The diesel particles were prepared as suspensions both with and without ferric oxide as described under Materials and Methods. Hamsters received 0.2 ml containing the indicated levels of DP or DP-Fe_2O_3 with the exception of the 10 mg dose groups which received two 5 mg treatments weekly. Surviving hamsters were sacrificed five weeks following the 15-week treatment period and selected tissues taken for histologic processing.

Lung Clearance of Diesel Particles

A limited study of lung clearance of diesel particles was carried out on a small group of hamsters. Each animal was administered a single dose of 5 mg DP by intratracheal instillation and pairs of animals were killed at 1 hour, 8 days, 30 days and 60 days post treatment. The lungs and trachea of one animal at each time period were photographed and lung and trachea from the second animal were processed for histopathologic examination.

Figure 1. Experimental Design for Dose-Range Study to Determine Toxicity of Diesel Fuel Extract Using the Localized Intratracheal Instillation Technique.

Group	No. of Hamsters	Treatment
1	25	Solvent (10% DMSO, 10% ETOH, 20% PG, 60% saline)
2	50	1.00% (5.00 mg) DFE
3	50	0.50% (2.50 mg) DFE
4	50	0.25% (1.25 mg) DFE
5	10	Saline
6	10	Shelf Control

Male Syrian Golden hamsters (Engle Labs.) received the initial instillations at 7 wks of age.

Volume of solution delivered at each instillation was 0.5 ml.

Animals were treated 1X/wk for 10 wks.

All animals which survived the treatment period were sacrificed at 12, 15 and 25 wks after the initial treatment.

Figure 2. Experimental Design for Dose-Range Study to Determine Toxicity of Diesel Fuel Particles Using the Saffiotti Intratracheal Instillation Technique.

Group	No. of Hamsters	Treatment	Frequency
1	50	5 mg DP	2X/wk
2	50	5 mg DP	1X/wk
3	50	3 mg DP	1X/wk
4	50	1 mg DP	1X/wk
5	50	5 mg DP + 5 mg Fe_2O_3	2X/wk
6	50	5 mg DP + 5 mg Fe_2O_3	1X/wk
7	50	3 mg DP + 3 mg Fe_2O_3	1X/wk
8	50	1 mg DP + 1 mg Fe_2O_3	1X/wk
9	50	3 mg BP + 3 mg Fe_2O_3	1X/wk
10	50	1.5 mg BP + 1.5 mg Fe_2O_3	1X/wk
11	50	5 mg Fe_2O_3 in solvent	2X/wk
12	50	Solvent, 0.5% gel-saline, 7% PG	2X/wk
13	25	Shelf Controls	NONE

Male Syrian Golden hamsters (Engle Labs.) received the initial instillation at 12 wks of age.

Volume of suspension delivered at each instillation was 0.2 ml.

All animals received 15 wks of treatment and survivors were sacrificed 5 wks after the final treatment.

SECTION 4

RESULTS AND DISCUSSION

LOCALIZED TREATMENT WITH DFE (SCHREIBER METHOD)

A series of preliminary experiments were carried out on the dichloromethane extract of diesel emission particles. The catheter design used in this technique requires that the test material be in solution and of relatively low viscosity. A number of solvent mixtures containing varying quantities of PG, DMSO, ethanol, and saline were tried as appropriate solvents in this system. Suitable dosage forms containing up to 1% DFE were prepared and evaluated for toxicity. Three preliminary experiments were carried out in which weekly treatments were carried out for ten weeks (Experiments 1 and 2) and for fifteen weeks (Experiment 3). The results of these experiments, summarized in Table 4, indicate no untoward effect on weight gain or on mortality that can be attributed to treatment with DFE.

Although it appeared possible therefore to prepare and test extracts by this technique it was decided not to continue with studies using this method of treatment. The localized treatment method cannot be used with particle suspensions so no direct comparison of the carcinogenic potential of DP and DFE can be carried out. In addition this technique has not been widely used and it has not been demonstrated that the trachea is responsive to the classes of combustion products that are present in DFE. Thirdly the total dose of test material delivered to the hamster cannot be readily determined in this procedure. The concentration of test material to which the test animals are exposed is known, but how much of the test material is retained by the animal following the reabsorbtion phase of the interbation cycle is uncertain.

Thus it was concluded that while the method is of potential interest as a research tool, program emphasis was to be placed on carcinogenicity trials using the better established intratracheal instillation method of Saffiotti.

TABLE 4. PERCENT INCREASE IN BODY WEIGHT AND PERCENT DEATH IN HAMSTERS ADMINISTERED DIESEL FUEL EXTRACT BY LOCALIZED INTRATRACHEAL INSTILLATION

Treatment	Experiment 1		Experiment 2		Experiment 3	
	% Increase in Body Weight	% Death	% Increase in Body Weight	% Death	% Increase in Body Weight	% Death
Solvent[a]	66	0[b]	81	8	88	0
1.0%, DFE	66	8	76	6	85	4
0.5%, DFE	50	0	59	10	87	6
0.25%, DFE	35	12	63	2	84	6
Saline	N.A.	N.A.	63	10	96	0
Shelf Control	49	0	62	0	92	0

[a]Solvent varied for each experiment.

[b]Percent of spontaneous death during 6 months of holding.

DOSE RANGE STUDY OF DIESEL EMISSION PARTICLES

An experiment was carried out to provide information for selection of doses for the chronic study of diesel particles by the Saffiotti method. Diesel particles and DP-Fe_2O_3 mixtures were prepared by ball milling as detailed in Materials and Methods. Hamsters were treated at weekly doses of 1, 3, 5, and 10 mg. Animals at the 10 mg weekly level were treated twice weekly with doses of 5 mg each. Hamsters were held for 5 weeks following the 15 weeks of treatment, survivors were sacrificed, and tissues were processed for histopathologic examination.

Table 5 shows the results obtained in this experiment in terms of mortality and percent increase in body weight over the twenty weeks of experiment. No treatment associated increased mortality was seen with the exception of the 3 mg benzo(a)pyrene positive control group which produced a high degree of lethality and a decreased weight gain. Twice weekly treatments with 5 mg DP or with 5 mg DP admixed with 5 mg of Fe_2O_3 led to a decreased weight gain as compared to control animals also receiving twice weekly instillations.

Microscopic examination of histologic sections of hamsters on this study led to the following conclusions.

1. There was no significant non-respiratory tract pathology in treated animals.

2. Lesions of the lungs were common in treated animals and included these findings:

   a. Adenomatous hyperplasia is more severe and extensive in DP treated animals than in vehicle control animals.

   b. Adenomas are more numerous and larger in the DP treated animals than in the vehicle control.

   c. Metaplasia to ciliated epithelium and squamous metaplasia occurred in some DP treated animals but not in vehicle controls.

   d. Severe multifocal reactive pneumonitis with hyperplasia and evidence of atypia occurred in DP treated animals but did not occur in vehicle controls.

TABLE 5. DOSE-RANGE STUDY TO DETERMINE THE TOXICITY OF DIESEL FUEL PARTICLES ADMINISTERED BY THE SAFFIOTTI TECHNIQUE

Treatment	No. of Animals	TREATMENT FREQUENCY			
		ONCE WEEKLY		TWICE WEEKLY	
		Percent Mortality	Percent Increase in Body Weight	Percent Mortality	Percent Increase in Body Weight
5 mg DP	50	6	14	6	8
3 mg DP	50	2	14	-	—
1 mg DP	50	6	14	-	—
5 mg DP:5 mg Fe_2O_3	50	6	11	10	10
3 mg DP:3 mg Fe_2O_3	50	4	10	-	—
1 mg DP:1 mg Fe_2O_3	50	4	10	-	—
3 mg BP:3 mg Fe_2O_3	50	38	7	-	—
1.5 mg BP:1.5 mg Fe_2O_3	50	12	10	-	—
5 mg Fe_2O_3	50	-	—	6	13
Solvent	50	-	—	10	13
None	25	0	9	-	—

The degree of severity and frequency of occurance of hyperplastic, metaplastic, and dysplastic changes in lung tissue of hamsters given twice weekly treatments of 5 mg DP each plus the decreased weight gain observed in these treatment groups led to the conclusion that the high dose selected for the chronic study would be 5 mg of diesel particles, once weekly, for 15 weeks.

LUNG CLEARANCE OF DIESEL PARTICLES

Hamsters were treated with a single instillation of 5 mg DP and pairs of animals were sacrificed at time periods of 1 hour, 8 days, 30 days and 60 days post treatment. Color photographs were taken of *en bloc* and exploded views of the lungs and trachea from one animal of each pair. The respiratory tract of the other animal was processed for histopathology.

Photographic reproductions of the lungs and trachea of animals in this study were included in IITRI Reports L6109-1 and L6109-2. These photographs showed that the particles were dispersed throughout the lung tissue as well as the trachea at one hour post instillation. Eight days after the instillation the particles were still very prevalent in the lungs, but appeared to be mostly cleared from the trachea. At 30 days post instillation, the particles remained heavily concentrated in the lungs. Gross observation of a hamster sacrificed 60 days post instillation showed particles still remaining in the lung but in apparently reduced amounts.

Lungs and tracheas of additional hamsters killed at these four time periods following a single administration of 5 mg DP were also processed for histopathologic examination.

A summary of the histopathologic findings on these animals follows.

79-393 DP 1 Hr.

- Lung---

There were numerous regions with diesel particles within the alveoli, bronchioles and bronchi. The diesel particles varied in form and in size and were found in spaces and upon the surfaces of the structures of the lung. There was no reaction to the diesel particles by the lung tissues.

- Trachea---

Diesel particles were found upon the mucosal surface.

79-395 DP 8 Days

- Lung---

Macrophages were present in numerous places within alveolar spaces and within septa. Within the macrophages there were diesel particles and extracellularly also there were diesel particles. Some of the diesel particles were on the epithelial surface of the alveolar septa. The internal structure of most of the macrophages was not visible because of the density of the diesel particles, however some macrophages did have one to several nuclei visible. The alveolar and bronchiolar epethelium was hyperplastic and proliferating in several places where there were diesel particles and macrophages. These lesions, focally, combined to form adenomatoid and papillomatoid structures.

- Trachea---

The diesel particle material had penetrated, in small amounts, into the submucosa.

79-397 DP 30 Days

- Lung---

In many places there were macrophages within alveolar spaces and septa; within the macrophages there were diesel particles. The alveolar and bronchiolar epithelium, in association with the macrophages bearing particles was hyperplastic and proliferating in several loci. The macrophages were less numerous than in the lung tissue from case 79-395 (see above).

- Trachea---

Similar to that seen in case 79-395 (see above).

79-520 DP 60 Days

- Lung---

Similar to some extent to case 79-397 except that there was more hyperplasia and proliferation of the alveolar and bronchiolar epithelium. There was also hyperplasia and proliferation of the bronchial epithelium.

- Trachea---

Similar to that seen in case 79-395.

The study demonstrated that the Saffiotti method of intratracheal instillation led to good dispersion of the diesel particles throughout alveoli, bronchioles, and bronchi. Although no quantitative estimates of clearance rates can be obtained the photographic representations and tissue sections both showed that diesel particles were still present in good number even 60 days after a single instillation of 5 mg of DP.

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APPENDIX A

SAFETY PLAN FOR HANDLING OF CARCINOGENS AND TEST MATERIALS

General safety precautions for personnel handling the carcinogens benzo-(a)pyrene (BP) and methylnitrosourea (MNU) will be in accord with the overall IITRI safety program which meets or exceeds Federal (OSHA) and State Standards. This program is under the overall jurisdiction of the IITRI Safety Officer. The IITRI medical center, which is staffed by an industrial health R.N., will be available for health problems and will keep complete records on all staff members involved in handling the carcinogens.

Preparing and Handling Methylnitrosourea

MNU is highly toxic, explosive, and a very potent carcinogen. It is sensitive to light, heat, high pH, and moisture.

1. The chemical, will be kept at -70^{o} in a sealed container until subdivided.

2. Before being opened and weighed, the chemical will be removed from the freezer and defrosted at room temperature.

3. The container will be opened in a safety hood maintained under negative pressure and subdivided into smaller containers.

4. Multiples of four bottles, housed in a larger container with desiccant, will be refrozen at -70^{o}.

5. In preparation for intratracheal injection, the following steps will be carried out in a negative pressure hood:

    a. Weigh Erlenmeyer flask.
    b. Weigh MNU.
    c. Add MNU to flask and weigh both as a check on the weight of MNU.
    d. Add vehicle (steroid suspending solution) to achieve stock concentration.
    e. Cover flask with parafilm.
    f. Heat to $30^{o}C$ in a water bath for 15 min to solubilize MNU.
    g. Dilute stock to achieve injecting concentration.

6. In all instances, two buckets of sodium carbonate solution (pH $\geq$ 10) will be present and, should any portion of the body or personal attire be contaminated, the worker will IMMEDIATELY flood himself with sodium carbonate solution.

Preparing and Handling of Dimethylbenz(a)anthracene

DMBA is a potent carcinogen and will be handled using the same precautions as described for MNU.

Preparing and Handling Benzo(a)pyrene in Ferric Oxide

BP is a potent carcinogen and is relatively insensitive to light, temperature, moisture, or pH. It is solubel in nonpolar solvents and insoluble in water. For administration to hamsters, the BP will be combined with Fe_2O_3 as a nucleated preparation.

1. The dry chemical will be stored in the refrigerator for not longer than six weeks.

2. To prepare for intratracheal injection, the following steps will be carried out in a negative pressure hood:

   a. Weigh Erlenmeyer flask.
   b. Weigh BP-Fe_2O_3
   c. Add carcinogen to flask and reweigh as a check on the weight of BP-Fe_2O_3.
   d. Add gelatin-saline vehicle and stopper flask.
   e. Vortex for 10 min.

Intratracheal (IT) Administration of Carcinogens

1. During all IT procedures, suspenion and solutions will be covered at all times except when supplemental vortexing is performed (e.g., with BP-Fe_2O_3 suspensions).

2. Laboratory counter tops will be covered with absorbant paper backed with a plastic sheet.

3. The cover from the flask containing the carcinogen will be removed and an aliquot withdrawn into a 0.25 ml syringe.

4. After the carcinogen has been administered to the animal, the IT needle will be replaced and the next animal treated. Both syringe and needel will be replaced after six hamsters have been treated.

5. All treated animals and their cages will be handled only by persons wearing gloves, masks, and laboratory coats.

Cleanup Procedures

1. MNU

   a. All instruments and glassware used in preparing and administering MNU will be placed in sodium carbonate solution (pH > 10) for at least 18 hrs, washed with soap and dried for reuse.

   b. All used gloves, masks, contamination mats, and all other disposable material will be placed in sodium carbonate solution overnight. These will then be bagged in plastic and incinerated.

2. BP-Fe_2O_3

   a. All instruments and glassware will be soaked in 1% Alconox^R solution for at least 1 hr, rinsed and sonicated in fresh Alconox solution, rinsed in distilled water and dried for reuse.

   b. All gloves, masks, contamination mats, and other disposable material will be bagged in plastic and disposed of by incineration at 1800^{o}F.

3. DMBA

   a. Bag and incinerate all waste and disposables.

Personnel Considerations

1. All personnel will wear surgical gloves, face masks, and laboratory coats.

2. All project team members will be instructed in handling and safety precautions.

3. Procedures and Precautions will be posted in appropriate places in the laboratory.

4. New personnel will be given safety training before joining the program.

5. The Principal Investigator will initially and periodically monitor operations to ensure adherence to all protocols.

APPENDIX B

PROTOCOL FOR THE COLLECTION AND SUBMISSION OF NECROPSY SPECIMENS (L6109)

INTRODUCTION

Prior to sacrifice of animals on project L6109 the following procedures will be used:

1. Final weighing of animal.

2. Animal necropsy forms completed with accession number.

3. Specimen jar labeled with experiment, animal group data, and accession number written on a small piece of card, and placed in the jar and on specimen jar tape.

PROCEDURES FOR NECROPSY EXAMINATION

Anesthetic and Verification Station (1 Person)

Animals will be anesthetized in a CO_2 chamber. After the animal is anesthetized, it is removed from the chamber. This animal with its necropsy form and specimen jar is carried to the necropsy examination station. After the necropsy has been completed the necropsy form and specimen jar is returned to verifier, for verification, before the animal carcass is discarded.

Necropsy Examination Station (2 to 3 Persons)

Person performing the necropsy examination will record the gross observation findings on the necropsy form. All pertinent information concerning the animal must be recorded in the information block at the top of the form. No pathologic diagnosis will be made to gross; all abnormalities will be described as to location, color, consistency and size. The pathologist will be called to view all unusual lesions.

Pin the animal to the dissecting board exposing the ventral surface. Disinfect the area with 70% alcohol. Using scissors rapidly make a "V" shaped incision through the skin and muscle layers extending from the pubis to each axilla (Observe all organs).

Dissect the trachea, larynx, esophagus and lungs *en mass*. Inflate the lungs via intratracheal perfusion with 10% formalin, attach a paper clip to trachea before placing the lungs and accompanying tissue into formalin.

Turn the animal over and remove the skin from the head including the fleshy tip of the nose, examine the nasal region for bulges or deviation, submit the intact maxillary region to histology for sectioning.

When the gross necropsy examination is completed, the form should be signed, dated, and returned with the specimen jar to verification station.

1. Anesthetic and Verification (Charlotte Nicholes)

2. Necropsy Examination (Edward Cichocki)
(Mary Ann Phee)
(Lawrence Dooley)

ORGAN CHECK LIST

Accession No. 79-

Project No. L6109

	T/N	NN
EXTERNAL SURFACE/ORIFICES		
AXILLARY LYMPH NODE	NR	NR
SKIN - ABDOMINAL		
SALIVARY GLAND-SMAX.		
LYMPH NODE - MANDIB.		
MAMMARY GLAND		
PERITONEAL CAVITY		
STERNUM		
MARROW SMEAR-FEMUR		
COSTO-CHONDRAL JUNCTION	NR	NR
PLEURAL CAVITY		
TONGUE	NR	NR
THYMUS		
THYROIDS		
HEART *		
ESOPHAGUS		
PARATHYROIDS		
LUNGS *		
LARYNX		
TRACHEA		
RESPIRATORY NODES		
AORTA		

	T/N	NN
LIVER *		
GALL BLADDER		
SPLEEN *		
PANCREAS		
PUBLIC SYMPHYSIS		
OVARIES	NR	NR
UTERUS	NR	NR
VAGINA	NR	NR
TESTES *		
VAS DEFERENS	NR	NR
PROSTATE	NR	NR
URETHRA		
BLADDER		
STOMACH		
DUODENUM		
JEJUNUM		
ILEUM		
CECUM		
COLON		
RECTUM	NR	NR
MESENTERIC LYMPH NODE		

	T/N	NN
ADRENALS *		
KIDNEYS *		
URETERS		
MUSCLE		
SCIATIC NERVE		
EAR/TAG	NR	NR
EAR CANALS		
BRAIN *		
PITUITARY		
EYES		
NASAL TURB.		
SPINAL CORD - Thoracic with ribs		
SPINAL CORD-LUMBAR		
BLOOD SMEAR		
L_1		
L_2		
L_3		
L_4		
L_5		

T/N = Tissue taken at necropsy and is normal
NR = Not Required
NN = Tissue taken at necropsy and is not normal
L.N. = Lymph Node
* = Organs are to be weighed
Verified = Fixed tissues are checked to confirm the presence of the indicated organs or tissues.

Tissues collected by: _____ Date _____

Read and reviewed by: _____ Date _____

Verified by: _____ Date _____

OBSERVATIONS & WEIGHTS

Species Hamster (M) F Accession No. 79-

Study D Dose Animal No.

Project No. L6109 Age wk days Duration wk days

Project Leader Dr. Clinton Grubbs Died/(Sac) Date

Testes	g	Liver	g	Brain	g
Kidneys	g	Heart	g	Body Weight	g
Spleen	g	Lungs	g		g

Balance Operator

OBSERVATIONS

Prosector Read and reviewed by: Date

(✓) = Wet sectioner, call Pathologist